## REMARKS

Claims 1-16 were currently pending. Claims 2 and 3 are amended herein. Support for the amendments are found throughout the specification at, *inter alia*, page 16, 1-5 and the original claims. Therefore, it is believed that no new matter is added.

Claims 1-16 are pending. No claim is allowed.

**Rejection Under 35 U.S.C. § 102 (a)**

Claims 2, 3, 5, 7, 9-11, 13, and 14 are rejected under 35 U.S.C. § 102 (a) as allegedly being anticipated by Korbelik et al. as evidenced by Kresl et al. According to the Examiner, Korbelik teaches a method of treating tumors in a subject comprising administering effective amounts of a green porphyrin and an immunoadjuvant, namely a mycobacterial cell wall extract or live BCG vaccine, and irradiating the subject with light comprising a wavelength absorbed by said photosensitizer. The Examiner also asserts that Kresl provides evidence that mice implanted subcutaneously with EMT6 cells are at risk for developing tumors from metastasis of the primary tumor. Applicants traverse this rejection.

Applicants respectfully submit that Korbelik fails to teach or suggest each and every limitation of the claimed methods. Korbelik lacks any teaching or suggestion of use of the green porphyrin PDT in combination with an immunoadjuvant following the eradication or removal of the primary tumor. Korbelik's disclosure focuses exclusively on the treatment of a primary tumor. Moreover, Korbelik fails to teach or suggest the administration of the immunoadjuvant intratumorally or systemically (as acknowledged by the Examiner at page 7 and 8 of the Action dated April 9, 2004). Therefore, Korbelik lacks disclosure of each and every element of the claimed methods, failing to anticipate the methods of the current claims.

Accordingly, it is believed this basis for rejection may be withdrawn.

**Rejection Under 35 U.S.C. § 103 (a)**

Claims 1 and 6 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Korbelik et al. in view of U.S. Patent No. 5,095,030A (hereinafter "the '030 patent"). According to the Examiner, Korbelik does not expressly disclose the use of PDT and

immunoadjuvant to treat a subject with tumors that result from the metastasis of a primary tumor or the effective amount of photosensitizer as 0.05-1.0 mg/kg for the subject. Nonetheless, the Examiner argues that it would have been *prima facie* obvious to use this range because the '030 patent teaches amounts in that range are effective and one would be motivated to treat cancer in patients diagnosed with a primary tumor or at risk for developing metastases of a metastatic primary tumor.

Claims 1 and 4 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Korbelik et al. in view of Momma et al. Again, the Examiner acknowledges that Korbelik lacks any disclosure regarding the use of the claimed method to treat the metastasis of a primary tumor while also acknowledging that Korbelik is silent regarding the use of such a treatment in a person previously undergone cancer or tumor therapy. The Examiner asserts that it would have been *prima facie* to one of ordinary skill in the art to treat a subject with a tumor arising from a metastasis of a primary tumor.

Claim 9 is rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Korbelik et al. in view of US Patent No. 6,290,712 B1 (hereinafter "the '712 patent") or alternatively over Korbelik in view of the '030 patent in view of Momma et al. as applied to claim 1 above, and in further view of the '712 patent. The Examiner acknowledges that Korbelik lacks any teaching or suggestion that the immunoadjuvant can be administered intratumorally. The Examiner asserts that the "712 patent teaches the administration of immunoadjuvant intratumorally and emphasizes the combination of sensitizer and immune-stimulation as key for tumor treatment.

Claims 8 and 12 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Korbelik et al. in view of US Patent No. 5,579,554 (hereinafter "the '554 patent"), or alternatively over Korbelik in view of the '030 patent or Korbelik in view of Momma et al., as applied to the rejection of claim 1 above, and in further view of the '554 patent. According to the Examiner, the '554 patent teaches an aqueous mycobacterial cell wall extract that can be injected intratumorally or systemically. The Examiner argues that one of skill in the art would be motivated to practice the method of Korbelik using the mycobacterial cell wall extract of the '554 patent because the immunoadjuvant can be administered intratumorally or systemically.

Claim 15 is rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Korbelik et al. in view of US Patent No. 6,071,944A (hereinafter "the '944 patent"), or alternatively over Korbelik in view of the '030 patent or Korbelik in view of Momma et al., as applied to the rejection of claim 1 above, and in further view of the '944 patent. The Examiner acknowledges that none of the references expressly teach or suggest the use of an additional irradiation step where a light of a wavelength that increases the penetration of the wavelength of light absorbed by the photosensitizer is used. According to the Examiner, the '944 patent teaches the pretreatment of pigmented tumors with high peak power light that enhances their susceptibility to conventional photodynamic therapy.

Claim 16 is rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Korbelik et al. in view of Johnston et al. and US Patent No. 4,912,094 (hereinafter "the '094 patent"), or alternatively Korbelik in view of the '030 patent or Korbelik in view of Momma as applied to the rejections of claim 1 above, and in further view of Johnston and the '094 patent. The Examiner acknowledges that Korbelik lacks any teaching or suggestion that an immunoadjuvant comprising mycobacterial cell wall skeleton and de-3-acylated lipid A. The Examiner asserts that Johnston teaches an immunoadjuvant comprising purified mycobacterial cell wall skeleton and MPL. The '094 patent teaches that modified lipopolysaccharides retain immunostimulating capacity and have the advantage of being less toxic. Thus, one of skill in the art would be motivated to use these to treat cancer.

Applicants traverse these rejections.

As a preliminary matter, Applicants respectfully submit that one of ordinary skill in the art would not assume that a treatment that reduces or eliminates a primary tumor will necessarily be effective against metastatic disease. It is well known in the art that metastatic disease and its resultant tumors can differ in any number of characteristics from the primary tumor. See, e.g., Exhibit A (discussing the various changes that occur between the primary tumor and the metastatic tumor). Thus, while it may be obvious to try such an approach, for one of ordinary skill in the art there is no reasonable expectation of success without some evidence that metastatic disease did occur and was in fact resolved and/or mitigated by the treatment.

None of the cited combinations render the method of claim 1 (and related dependent claims) *prima facie* obvious as the primary reference of Korbelik lacks any teaching or suggestion of using green porphyrins in PDT with an immunoadjuvant to treat *metastatic* tumors in a subject, and therefore provides neither a motivation to modify Korbelik's teachings or a reasonable expectation of success for such a modification. Applicants again note that the Examiner acknowledges in the instant Action that Korbelik "does not expressly disclose the method can be used to treat in a subject, tumor that result from the metastasis of a primary tumor". *See* page 5 of Action dated April 9, 2004. This deficiency in Korbelik is not rectified by the many cited secondary references or any combination thereof.

The '030 patent fails to teach or suggest the use of PDT in combination with an immunoadjuvant to treat metastatic disease. The '030 patent discloses the use of PDT therapy alone to prevent the development of tumors as well as disclosing the dose range used in claim 6. However, there is no disclosure whatsoever suggesting that anything other than PDT is required to prevent the development of tumors. The disclosure of the cited dose is in reference to the use of PDT alone. A person of ordinary skill in the art would be well aware of possible toxicity that can result from the combination of therapies when used at doses that are effective for each agent alone. Without more, it is sheer speculation that the skilled artisan would look to this dose range as one that would be effective for the combination of PDT and an immunoadjuvant. Moreover, the '030 patent contains no disclosure that motivates the skilled artisan to extend the teachings of Korbelik to the treatment of metastatic disease. In fact, the teachings of the '030 patent conflict with those of Korbelik because the '030 patent discloses that PDT alone is sufficient. Therefore, the '030 patent fails to remedy the deficiency of Korbelik because it does not disclose that the use of PDT and an immunoadjuvant can be used to treat metastatic disease. To date, the Examiner has provided no scientific rationale showing that one of ordinary skill in the art would further modify a method already known to be effective.

Likewise, Momma contains no disclosure that motivates the skilled artisan to modify Korbelik to treat metastatic disease, therefore also failing to remedy Korbelik's deficiency. As with the '030 patent, Momma fails to teach the use of PDT with an immunoadjuvant to treat metastatic disease. In fact, Momma teaches that the use of PDT alone enhances the incidence of metastatic

disease in this model. *See, e.g.*, Momma et al., at Abstract ("The PDT-alone group, however, had a mean number of lung metastases per animal, which was nine times the control group and 34 times the combination group."). The authors merely suggest that more work should be done without offering any guidance that would point the skilled artisan to the use of an immunoadjuvant. *See, e.g.*, Momma et al., at page 5430 ("Future work will study the effects of varying treatment parameters ... to clarify the determinants required for favorable long-term response after PDT of prostate cancer."). The disclosure of marked reduction of metastatic disease with surgery and PDT by Momma actually *teaches away* from the claimed methods. The success of such a method provides no motivation to modify the PDT/surgery combination by including an immunoadjuvant. In fact, the data disclosed in Momma suggests that certain enhancements of PDT's anti-tumor effects may result in increased metastatic disease. Thus, the mere fact that these references can be combined is not enough to render the claimed methods *prima facie* obvious. MPEP § 2143.01 ("The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art suggests the desirability of the combination.") (citations omitted) (original emphasis).

The '712 patent also fails to render the claimed methods *prima facie* obvious because combining the patent with Korbelik or the other cited references changes the principle of operation of the '712 patent. As previously discussed, the '712 patent is directed to a discrete set of chromophores that are distinguishable from green porphyrins in that the '712 patent expressly discloses the desirability of using chromophores that mediate cell death through a photothermal effect. To modify the teachings of the '712 patent to use green porphyrins in the absence of appreciable heating of the target tissue changes the principle of operation for the '712 patent. If the combination of references results in a change of principle of operation, the combination is non-obvious. *See* MPEP § 2143.01 ("If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims *prima facie* obvious." (citations omitted)). To date, the Examiner has not addressed how such a modification is desirable in view of the scientific understanding at the time of filing.

The '554 patent, the '944 patent, Johnston, and 'the '094 patent fail to rectify the basic deficiency in Korbelik. None of these references provides any teaching alone or in any combination that results in the claimed methods of using a green porphyrin and an immunoadjuvant to treat metastatic disease.

In the absence of any teaching, disclosure, or suggestion regarding the use of green porphyrins that do not generate appreciable heat when activated by light to treat metastatic disease, the combination of references cannot provide a reasonable expectation of success for the modification required to result in the claimed invention.

Accordingly, it is believed this basis for rejection may be withdrawn.

**Rejection Under the Judicially Created Doctrine of Obviousness-Type Double Patenting**

Claims 1-16 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-21 and 28-33 of the copending Application Serial No. 09/756,687 for reasons of records. Applicants traverse this rejection.

Applicants acknowledge Examiner's indication that this rejection will be held in abeyance until such time that patentable subject matter is indicated.

## CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 273012011100. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: October 12, 2004

Respectfully submitted,

By _____
Laurie L. Hill, Ph.D.
Registration No.: 51,804
MORRISON & FOERSTER LLP
3811 Valley Centre Drive, Suite 500
San Diego, California 92130
(858) 720-7955

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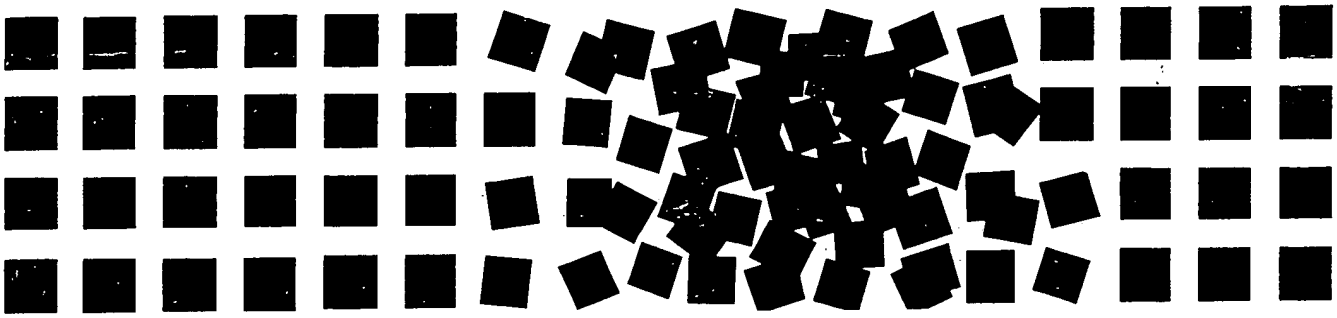

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6th edition

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**530 Walnut Street**
**Philadelphia, PA 19106 USA**
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Printed in the USA

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**Library of Congress Cataloging-in-Publication Data**
Library of Congress Control Number: 89-649-721
Cancer: principles and practice of oncology [edited by] Vincent T. DeVita, Jr., Samuel Hellman, Steven A. Rosenberg; 319 contributors.—6th
ISSN 0892-0567
ISBN 0-781-72387-6

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William G. Stetler-Stevenson
David E. Kleiner, Jr.

CHAPTER 8

# Molecular Biology of Cancer: Invasion and Metastases

Metastasis is the spread of cancer cells from a primary tumor to vital organs and distant sites in the cancer patient's body. Metastatic disease is the hallmark of malignant cancer. The formation of tumor metastasis is the major cause of treatment failure in cancer patients and is a principal contributing factor to cancer morbidity and mortality. Metastatic disease has significant biologic and prognostic impact on cancer diagnosis and treatment.

The formation of metastatic foci is a continuous process that can begin early in the growth of the primary tumor and increases in frequency with tumor duration and tumor burden. Metastases may also disseminate to form new metastatic lesions in other organs (metastases will metastasize). The variation in size, age, dispersed anatomic location, and heterogeneous composition of metastases makes complete eradication of metastatic disease by currently available therapeutic strategies extremely difficult. The patient with metastatic disease succumbs to organ failure secondary to anatomic compromise caused by multiple metastases or to complications associated with systemic therapy directed against the metastatic disease. The design of more effective therapies to treat metastatic cancer requires better understanding of the molecular events and cellular processes that are involved in the process of metastasis formation.

## THE METASTATIC CASCADE

It is well established, from both clinical observations and mechanistic studies, that metastasis formation is an inefficient process. Studies have demonstrated that late events in metastasis formation are largely responsible for the inefficiency of this process. Large numbers of tumor cells and tumor cell clumps are shed into the vascular drainage system of a primary tumor.[1,2] It has been demonstrated experimentally that, after intravenous injection of highly metastatic tumor cells, only approximately 0.01% of these cells form tumor foci. The number of circulating tumor cells and tumor emboli correlate with the size and age of the primary tumor (i.e., larger tumors shed more tumor cells and emboli). However, the number of circulating tumor cells does not correlate with the clinical outcome of metastases.

The inefficiency of tumor cells in completing the metastatic cascade is the result of the fact that successful formation of metastatic foci consists of several highly complex and interdependent steps. Each step is rate limiting in that failure to complete any of these events completely disrupts metastasis formation. The steps involved in metastasis formation are thought to be similar in all tumors and are characterized as follows (Fig. 8-1):

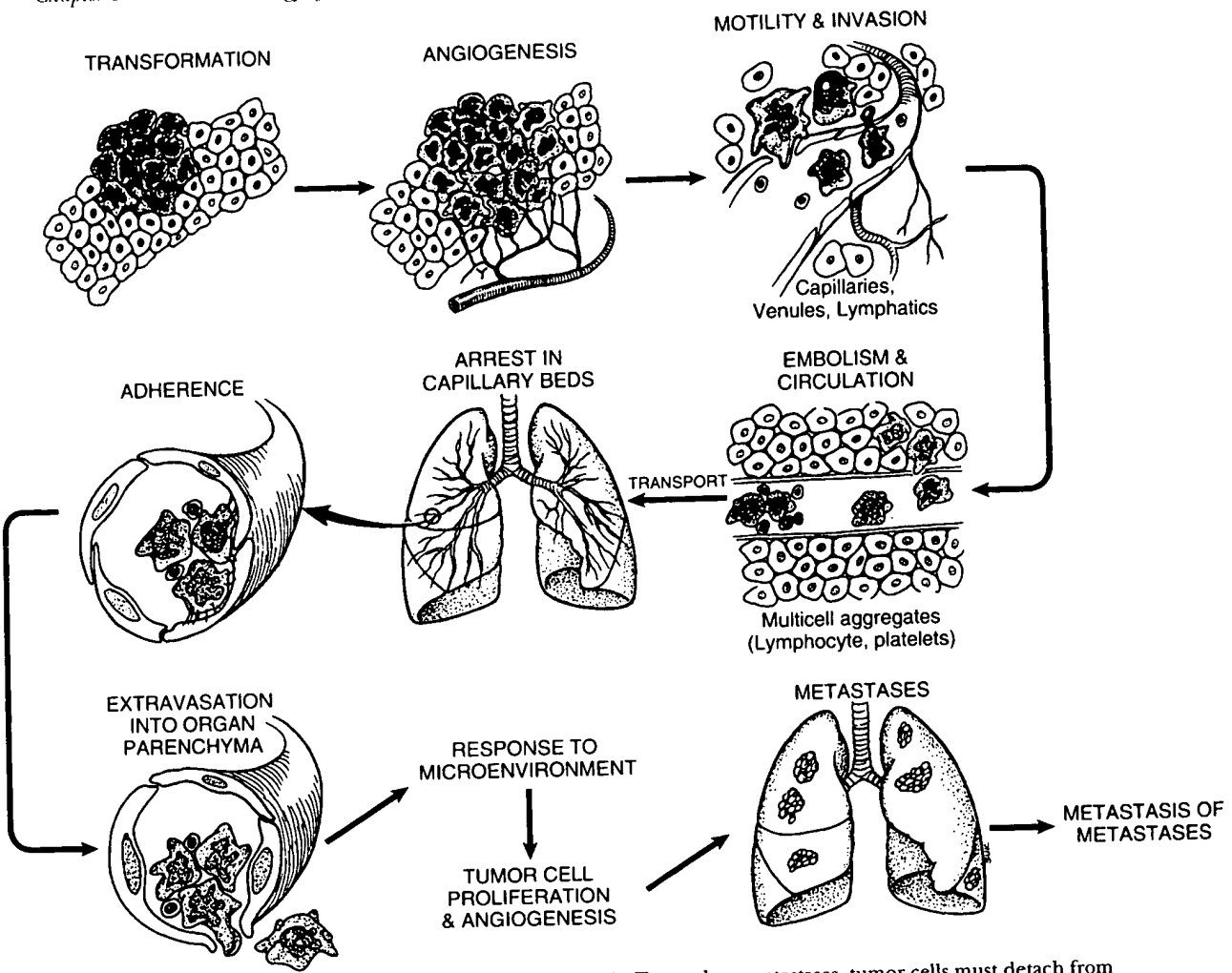


**FIGURE 8-1.** The pathogenesis of cancer metastasis. To produce metastases, tumor cells must detach from the primary tumor, invade the extracellular membrane and enter the circulation, survive in the circulation to arrest in the capillary bed, adhere to subendothelial basement membrane, gain entrance into the organ parenchyma, respond to paracrine growth factors, proliferate and induce angiogenesis, and evade host defenses. The pathogenesis of metastasis is therefore complex and consists of multiple sequential, selective, and interdependent steps whose outcome depends on the interaction of tumor cells with homeostatic factors.

1. Oncogenesis (tumorigenesis). After the initial neoplastic transformation, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential. The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate limiting for further growth.
2. Angiogenic switch. As the tumor grows and central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply. This process is called the *angiogenic switch* and involves the secretion of various angiogenic factors and the removal or suppression of angiogenesis inhibitors. Vascularization of the tumor is also associated with a dramatic increase in the metastatic potential of these tumors. Studies demonstrate that some tumors and metastases arising in vascularized tissues may first co-opt existing vasculature before initiating recruitment of new vessels.
3. Clonal dominance and invasive phenotype. Continued genetic alterations in the tumor cell population results in selection of tumor cell clones with distinct growth advantage and acquisition of an invasive phenotype. Invasive tumor cells down-regulate cell-cell adhesion, alter their attachment to the extracellular matrix (ECM) by changing integrin expression profiles, and proteolytically alter the matrix. Collectively, these changes result in enhanced cell motility and the ability of these invasive cells to separate from the primary tumor mass. These cells can detach from the primary tumor and create defects in the ECM that define tissue boundaries, such as basement membranes, thus accomplishing stromal invasion. Furthermore, the poorly formed tumor vasculature that forms in response to the angiogenic switch in phenotype of the primary tumor, as well as thin walled lymphatic channels in the surrounding stroma, are readily penetrated by these invasive tumor cells and offer ready conduits to the systemic circulation. Endothelial cells responding to the angiogenic stimulus produced by the primary tumor also express an invasive phenotype.

4. Survival in the circulation. Once the tumor cells and tumor cell clumps (emboli) have reached the vascular or lymphatic compartments, they must survive a variety of hemodynamic and immunologic challenges. Little is known about how these factors may impact on the inefficiency of the metastatic process in human cancers.
5. Tumor cell arrest. After survival in the circulation, tumor cells must arrest in distant organs or lymph nodes. This arrest may occur by size trapping on the inflow side of the microcirculation, or by adherence of tumor cells through specific interactions with capillary or lymphatic endothelial cells, or by binding to exposed basement membrane.
6. Extravasation and growth at the secondary site. In most cases, arrested tumor cells extravasate before proliferating. Studies suggest that extravasation of tumor cells is not dependent on protease activity and is independent of metastatic potential. After exiting the vascular or lymphatic compartments, metastatic tumor cells may proliferate in response to paracrine growth factors or become dormant. After extravasation, tumor cells migrate to a local environment more favorable for their continued growth. Findings using *in vivo* videomicroscopy demonstrate that the poor growth of tumor cells after extravasation from the circulation is a major factor contributing to the inefficiency of the metastatic process.
7. Angiogenesis in metastatic foci. Finally, continued growth of the metastatic foci is also dependent on angiogenesis. The development of this neovascular network at the metastatic site enhances the metastatic potential of these cells just as it does for the primary tumor.
8. Evasion of immune response. Metastatic foci of tumor cells must evade eradication by immune responses that may be either nonspecific or targeted directly against the tumor cells.

As might be expected from the highly complex nature of metastasis formation, no single gene product is responsible for metastasis formation. Successful completion of many of the steps of the metastatic cascade is the result of both the acquisition of positive effectors as well as the loss of negative regulators. Unrestrained growth is not sufficient to result in tumor invasion and metastasis. Tumor invasion is not a passive process secondary to tumor growth, and it may require additional genetic changes other than those associated with the tumorigenic phenotype. Tumorigenicity and metastatic competence have some overlapping features but are clearly under separate genetic control. Research on tumor cells has identified gene products that can facilitate completion of each of the steps outlined above. These are the molecular effectors of tumor invasion and metastasis. In many cases, research also has identified gene products that function to block successful completion of each of the steps in the metastatic cascade. The idea that there is loss of negative effectors, as well as positive phenotypic changes, associated with malignant progression and metastasis formation is now well established. In this chapter, some of the steps in the metastatic cascade are examined with the aim of identifying both the molecular mechanisms and the effector and suppressor genes that may become a target for new and effective cancer therapies. Immune modulation of cancer is discussed elsewhere (see Chapter 4), as is the process of tumor-associated angiogenesis (see Chapter 9). In addition, the angiogenic response at the metastatic foci is conceptually the same as in the primary tumor; therefore, the discussion of these points is limited here.

## ONCOGENESIS: METASTASIS AND TUMORIGENESIS ARE UNDER SEPARATE GENETIC CONTROL

Many tumors progress through distinct stages that can be identified by histopathologic examination. Pathologists use the identification of these distinct phases to diagnose and classify tumors into different prognostic categories (e.g., normal tissue, hyperplasia, dysplasia, carcinoma *in situ*, and frankly invasive carcinoma). Genetic analysis of these different stages of tumor progression resulted in the multistep theory of tumorigenesis, which involves activation of oncogenes, inactivation of tumor suppressor genes, and identification of a host of tumor-associated molecules (cancer markers). The progressive alteration in cellular oncogenes and inactivation of tumor suppressor genes that results in uncontrolled growth and loss of contact-dependent cell growth has been well documented in many tumor types, and it is the subject of separate chapters in this text (see Chapters 1 and 2). It also has been demonstrated that transfection of certain of these oncogenes into the correct recipient cell can result in acquisition of invasive and metastatic phenotype. Thorgeirsson et al.[3] were first to demonstrate that transfection of activated Ras oncogene sequences into fetal mouse fibroblasts result in acquisition of a metastatic phenotype when these cells are implanted in the nude mouse. This finding has been confirmed in both fibroblast and epithelial cells of human and rodent origin when transfected with Ras. Similar findings, but with lower efficiency, have been observed with several other oncogenes (Mos, Raf, Src, Fes, and Fms) when transfected into an *appropriate* recipient cell.

At first, these results might suggest that the metastatic phenotype might arise from genetic alterations associated with the acquisition of tumorigenicity. However, cells can be transformed by oncogene transfection, but not all cells acquire a metastatic phenotype after oncogene transfection.[4] In addition, the adenovirus 2E1A gene was shown to suppress Ras induction of the metastatic phenotype without alteration in tumorigenicity or inhibition of soft agar colony formation.[5] These findings demonstrate that a clear separation exists between the genetic changes that drive tumorigenicity and the metastatic phenotype. These findings can be explained by the fact that invasion and metastasis require activation of additional effector genes or suppression of local inhibitors over and above those required for uncontrolled growth alone. The failure to induce metastatic competence by Ras in some cell systems is explained by a deficiency or suppression of these effector molecules in these cell types. These metastasis effector genes or gene products may be downstream of Ras or totally independent pathways that are involved in cell attachment or regulation of protease activity. In studies in which Ras effectively confers a metastatic phenotype, the metastasis effector genes are activated and suppressor genes are inactivated before Ras transfection, and the addition of Ras oncogene allows completion of the metastatic cascade. This finding is demonstrated by the observation that Ras plus E1A reverses the metastatic potential and metalloproteinase expression observed in rat embryo cell lines.[5,6] Candidate effector genes include those

associated with cellular adhesion to ECM components, as well as proteases, motility factors, angiogenic factors, and growth regulation. Candidate suppressor genes would include genes associated with enhanced cell-cell attachment; phosphatase activities that regulate focal adhesion assembly; angiogenesis inhibitors; protease inhibitors; and factors that suppress cell migration, invasion, and growth.

## ANGIOGENESIS: BALANCE OF POSITIVE AND NEGATIVE EFFECTORS

Classic studies by Folkman and colleagues in the 1970s demonstrated that continued growth of tumors requires persistent new blood vessel growth and that inhibition of this angiogenic response results in tumor dormancy.[7,8] These studies suggested that tumor cells release soluble factors that induce the angiogenic response (i.e., angiogenic factors). This process is now referred to as the *angiogenic switch*—that is, the switch from a nonangiogenic to an angiogenic phenotype.[9]

It has been experimentally demonstrated that shedding of malignant cells into the venous drainage is commensurate with the initiation of a tumor angiogenic response and is quantitatively related to the surface area of new tumor vessel.[10] This process may be facilitated by the immature nature of newly formed tumor vessels. Weidner and colleagues have reported the correlation of angiogenic response with the frequency of metastasis, disease recurrence, and reduced survival in a number of different human tumors.[11–13]

Some reports challenge the presumption that most tumors and metastases originate as small, avascular tumor cell clumps that must recruit new vessels for continued growth. Studies find that tumors arising or metastasizing into vascularized tissues may initially co-opt existing blood vessels.[14] Subsequently, there is regression of the co-opted vessels and increased production of angiogenic factors by the tumor cells that recruit a neoangiogenic response.

Researchers have begun to identify angiogenesis inhibitors that either must be modified or removed during the tumor-induced angiogenic response. Examples of these inhibitors include platelet factor-4, interferon-α, thrombospondin, and protease inhibitors [tissue inhibitors of metalloproteinases (TIMPs)]. The list of inhibitors of angiogenesis continues to grow with the identification of pigment epithelium–derived factor.[15] Inhibitors of angiogenesis can work at many levels by blocking endothelial cell growth, cell attachment, or migration.

A novel direction in angiogenesis inhibitors is the identification of cryptic inhibitors that are revealed by proteolytic modification of ECM components. This finding suggests a class of angiogenesis inhibitors that are contained within other proteins that are not antiangiogenic and must undergo proteolytic processing to uncover the antiangiogenic activity. Examples of these types of inhibitors include a 29-kD fragment of fibronectin[9]; the 16-kD fragment of prolactin; angiostatin released from plasminogen[16,17]; and endostatin, a C-terminal fragment of collagen XVIII.[18] The newest member of this group is the cleaved conformation of the serpin antithrombin.[19] Thus, local regulation of angiogenesis can be achieved by proteolytic release of cryptic angiogenesis inhibitors.

In a study by Bergers et al.,[20] the RIP1-Tag2 model of pancreatic islet β-cell tumors has been useful in examining the effects of angiogenesis inhibitors on multistage carcinogenesis in mice. The findings of this study demonstrate that antiangiogenic drugs can be targeted to selective stages of cancer progression and that combination of agents with different mechanisms of action should be used at different stages of tumor development.

Tumor angiogenesis is the result of a tightly regulated process and a delicate balance of both pro- and antiangiogenic factors. The process of endothelial cell invasion and formation of new blood vessels has many functional similarities to the process of tumor cell invasion.[21] Understanding the mechanisms of cell invasion may allow identification of new strategies to disrupt the invasive process. In addition to therapeutic use of antiangiogenic agents, it should be possible to target molecules that are involved in the process of cellular invasion that is required for both angiogenesis and tumor metastasis.

## TUMOR HETEROGENEITY AND CLONAL DOMINANCE

It is now well recognized that most neoplasms consist of several tumor cell populations that vary widely in several important biologic characteristics. These characteristics include growth rates, karyotype, production of growth factors and stimulators of angiogenesis, hormone production, receptor content, and susceptibility to damage by cytotoxic agents, immune response, and hypoxia. Fidler et al.[22–24] first demonstrated the concept of heterogeneity of primary neoplasms in 1977. This concept is important because it suggested that not all tumor cells in the primary tumor population share the same propensity to form metastases and that formation of metastatic foci selects for an aggressive subpopulation of tumor cells out of the primary tumor. It was logical to assume that the size of the aggressive subpopulation in the primary tumor would reflect the propensity of that tumor to metastasize. This assumption would be of prognostic significance if a clinical assay of the primary tumor were able to detect and determine the presence of the highly aggressive subpopulation. However, evidence of a significant difference between the average metastatic propensity of cells comprising a primary tumor compared with those from an established metastasis was not forthcoming. This discrepancy was examined experimentally by Kerbel et al.[25–27] by using genetic markers to tag different subpopulations in the primary tumor. This work demonstrated that the metastatic subpopulation dominates the primary tumor mass early in its development. This dominance arises secondary to a selective growth advantage in the metastatic cells responding to local growth factors. Thus, the measurement of the average level of a molecular marker associated with metastatic propensity in the primary tumor reflects the likelihood of that tumor to form metastases. This finding has subsequently been confirmed in clinical studies by demonstration that the average level of a specific marker or oncogene measured within the primary tumor can be correlated with clinical evidence of metastasis or recurrence.

## DEFINING THE INVASIVE PHENOTYPE

During the transition from benign to invasive carcinoma, extensive changes occur in the quantity, organization, and distribution of subepithelial basement membrane. The hallmark of

invasive carcinoma is disruption of the epithelial basement membrane and the presence of cancer cells in the stromal compartment.[21,28] Benign proliferative disorders, such as fibrocystic disease, sclerosing adenosis, intraductal hyperplasia, intraductal papilloma, and fibroadenoma, are all characterized by disorganization of the normal epithelial architecture. But no matter how extensive this disorganization may become, these benign lesions are always characterized by a continuous basement membrane that separates the neoplastic epithelium from the stroma.[21,28]

In contrast, invasive carcinoma is characterized by a loss of basement membrane around the invasive tumor cells in the stroma. Once the basement membrane barrier is compromised, it is impossible to determine the quantity or location of tumor cells that may have escaped from the primary tumor. Thus, local invasion is paramount to malignant conversion.

The ability to cross basement membrane barriers is not unique to malignant carcinoma cells. During an inflammatory response, nonneoplastic immune cells regularly cross the subendothelial basement membranes, as do endothelial cells during the angiogenic response. Trophoblasts invade the endometrial stroma and blood vessels to establish contact with the maternal circulation during development of the hemochorial placenta. However, these normal cell types respond to additional signals that result in differentiation and subsequent loss of their invasive phenotype. Indeed, both normal and tumor cell invasion share functional similarities. In tumor cell invasion, however, it is the lack of response to negative signals or regulators and the continued invasion with ongoing tumor cell growth that is unique to cancer metastasis.[21,28]

Tumor cell interaction with the basement membrane is defined as the critical event of tumor invasion that signals the initiation of the metastatic cascade.[21,28] Basement membranes are composed of a dense meshwork of type IV collagen, laminin, and heparin sulfate proteoglycans that is interspersed with entactin and other minor components. Basement membranes do not contain pores that would allow passive tumor cell migration. Early studies on defining the invasive phenotype on malignant tumor cells focused on the interaction of tumor cells with the epithelial basement membrane. These studies defined the three-step hypothesis of tumor cell invasion: tumor cell attachment to the basement membrane, creation of proteolytic defects in the basement membrane, and migration of tumor cells through these defects.[21,28] It is now recognized that these three steps describe tumor cell interaction with all types of ECM and not just basement membrane. Furthermore, nonneoplastic invasive cells, such as trophoblasts, endothelial cells, and inflammatory cells, all use mechanisms for invasion that are functionally similar to tumor cells. The difference between these normal invasive processes and the pathologic nature of tumor cell invasion is therefore one of regulation. An understanding of the factors that control cellular processes essential to the invasive phenotype should allow identification of novel targets for therapeutic intervention to prevent and treat both angiogenesis and metastasis formation.

## CELL-CELL ADHESION SUPPRESSES OR FACILITATES METASTASIS FORMATION

The initial events in cellular invasion are changes in cell adhesion. These changes consist of alteration in both cell-cell adhesion and interactions with the ECM. A variety of cell surface receptors that mediate these interactions have been characterized, including the cadherins, integrins, immunoglobulin superfamily, and CD44. Tumor cells must decrease cell and matrix adhesive interactions to escape from the primary tumor. At later stages in the metastatic cascade, however, tumor cells may need to increase adhesive interactions with cells and ECM, such as during arrest and extravasation at a distant site. The apparent contribution of each class of cell adhesion molecules to invasive behavior will in some way be dependent on the tumor cell population and model system used to study these interactions. We review the contribution of changes in cell-cell adhesion to tumor progression before considering alterations in association with the ECM.

The majority of human cancers arise in epithelial cells. Several types of junctional structures, such as desmosomes, tight junctions, and adherens-type junctions tightly interconnect normal epithelial cells. The formation and maintenance of these contacts require $Ca^{2+}$-dependent homophilic interactions mediated by the cell-adhesion molecules known as *cadherins* (Fig. 8-2). Cadherins are a superfamily of single-pass transmembrane glycoproteins that mediate $Ca^{2+}$-dependent cell-cell adhesion. The cadherin superfamily now consists of five subfamilies: the classic type I and type II cadherins, desmosomal cadherins, protocadherins, and cadherin-related proteins.[29,30] The classic cadherin, epithelial cadherin (or E-cadherin), mediates homotypic cell adhesion in epithelial cells. E-cadherin is a transmembrane glycoprotein that has five extracellular homologous domains (ectodomains), a single membrane-spanning region, and a cytosolic domain. E-cadherin is physically anchored to the actin cytoskeleton by cytoplasmic proteins termed *catenins*. β-Catenin is also a major component of the WNT signaling pathway.

Any disruption of the intracellular E-cadherin–catenin complex results in loss of cell adhesion. This would include changes in E-cadherin expression or function, as well as genes other than E-cadherin that are required for complex formation and function. Abundant evidence indicates that E-cadherin function is frequently lost during progression of many human cancers, including those arising in the breast, prostate, esophagus, stomach, colon, skin, kidney, lung, and liver.[31,32] This loss of E-cadherin function arises via several different mechanisms. In familial gastric carcinomas, germline mutations in the E-cadherin gene predispose individuals to develop malignant cancer. Mutations in β-catenin are found in many primary tumors, including prostatic cancer, melanoma, and gastric and colon cancer. Another mechanism disrupting E-cadherin function during tumor progression is hypermethylation of the E-cadherin promoter, resulting in decreased gene expression. This has been found to be a major mechanism in papillary thyroid cancer in that 83% of cases of this disease demonstrated hypermethylation of the E-cadherin promoter.[33] Yet another mechanism to alter E-cadherin function is proteolytic modification. Lochter et al.[34] have reported that E-cadherin function can be disrupted by degradation of E-cadherin's extracellular domains by stromelysin-1, a member of the matrix metalloproteinase (MMP) family that has been closely linked with tumor progression. Constitutive expression of active stromelysin in mammary epithelial cells results in cleavage of E-cadherin and progressive phenotypic changes *in vitro*, including loss of catenins from cell-cell contacts, down-regulation of cytokeratins, and up-regulation of vimentin and MMP-9. These changes result in a stable epithelial to mesenchymal transition of

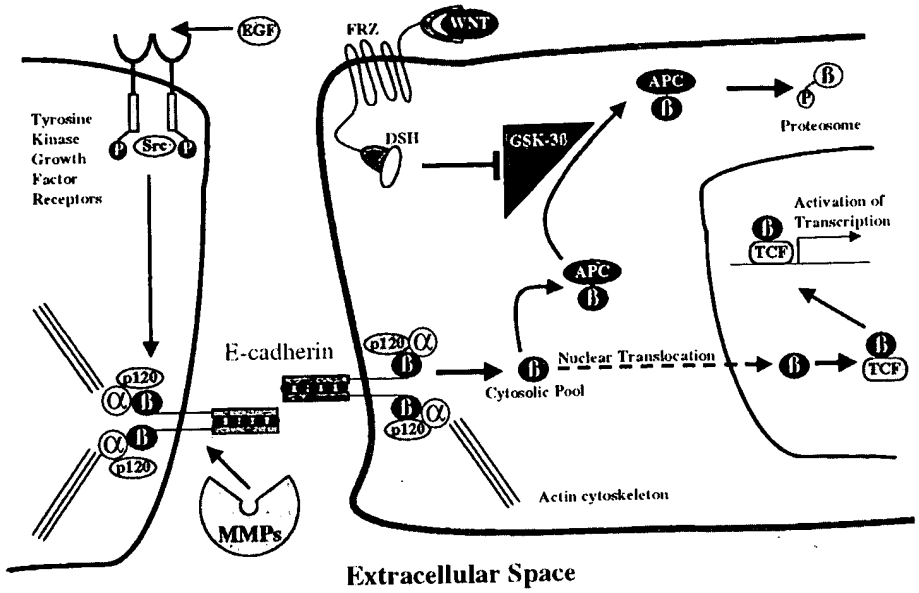


**FIGURE 8-2.** Disruption of cell-cell adhesion concomitant with tumor progression. E-cadherin is a homotypic cell adhesion molecule containing five homologous, extracellular domains (ectodomains) that bind divalent calcium ions. Calcium binding promotes homophilic cell-cell E-cadherin complexes found in such structures as desmosomes, tight junctions, and adherens-type junctions. The cytoplasmic tail of E-cadherin involved in cell-cell adhesion interacts with β-catenin, α-catenin, and $p120^{CAS}$ (p120). Loss of E-cadherin function, by germline mutation, promoter hypermethylation, or destruction of the ectodomains by matrix metalloproteinase (MMP) activity, results in an increase in free cytosolic β-catenin levels. Increased cytoplasmic β-catenin can be directed to the proteosome complex by glycogen-synthetase kinase 3β (GSK3β) phosphorylation and subsequent interaction with the adenomatous polyposis coli (APC) gene product. The frizzled (FRZ)-disheveled (DSH) pathway for WNT signaling can down-regulate the activity of GSK3β. Activation of the WNT signaling pathway or loss of APC function facilitate the increase in cytosolic β-catenin levels, which are associated with loss of E-cadherin function, or mutations of the β-catenin gene, which result in reduced association with E-cadherin cytoplasmic domain. Translocation of β-catenin to the nucleus results in gene expression associated with cell transformation and tumor growth (i.e., c-Myc, cyclin D1). It is noteworthy that this cascade of cellular transformation can be initiated by expression of an extracellular protease that culminates in enhanced chromosomal instability.[35] EGF, epidermal growth factor; P, phosphorylated amino acid residue(s); TCF, tissue coding factor.

cellular phenotype. *In vivo* stromelysin expression promotes mammary carcinogenesis that includes stereotyped genomic changes distinct from those seen in other mouse breast cancer models.[35] It has been reported that loss of H-cadherin expression occurs during the progression of breast cancer,[36] but little is known about the function of other cadherin family members during tumor progression. In summary, a decrease in cell-cell adhesion is associated with malignant conversion. Forced expression of E-cadherin in tumor cell lines results in reversion from an invasive to a benign tumor cell phenotype.[31,32]

In normal cells, β-catenin is sequestered in the intracellular adhesion complex with the cytoplasmic domain of E-cadherin, α-catenin, γ-catenin, and $p120^{CAS}$ (Crk-associated substrate). Loss of cell-cell adhesion results in disruption of the adhesion complex and free β-catenin. Free β-catenin is bound by the adenomatous polyposis coli (APC) gene product and is rapidly phosphorylated by glycogen-synthetase kinase 3β (GSK3β). Phosphorylated β-catenin is subsequently degraded in the ubiquitin proteosome pathway. In many colon cancer cells, the tumor suppressor gene APC is nonfunctional. This lack of function can lead to accumulation of high levels of cytoplasmic β-catenin that can subsequently be translocated to the nucleus. The WNT-1 protooncogene–initiated signaling pathway, which includes the frizzled (FRZ) and disheveled (DSH) gene products, can block activity of GSK3β, which can also result in accumulation of β-catenin. In the nucleus, free nonphosphorylated β-catenin can bind to members of the TCF (tissue-coding factor)/LEF-1 (lymphoid enhancer-binding factor 1) family of transcription factors. It has been demonstrated that, after inactivation of APC function, the increase in available β-catenin enters the nucleus, complexes with transcription factor Tcf-4, and up-regulates c-Myc expression.[37] It also has been shown that β-catenin activates transcription from the cyclin D1 promoter and contributes to neoplastic transformation by causing accumulation of cyclin D1.[38] These findings link changes in cell-cell adhesion with intracellular signaling, oncogene expression, and tumor cell growth. Thus, loss of cadherin-mediated cell-cell adhesion is an important event that has many far-reaching consequences for acquisition of the invasive phenotype and tumor progression.

Other types of cell-cell adhesive interactions can actually facilitate metastasis formation. These may be particularly important during tumor cell arrest and extravasation. These molecules include members of the immunoglobulin superfamily, such as nerve cell adhesion molecule and vascular cell adhesion molecule-1.[39–43] The immunoglobulin superfamily has a wide variety of members involved in cellular immunity and signal transduction, as well as cell adhesion. Members of the superfamily share the immunoglobulin homology unit that consists of 70 to 110 amino acid residues organized into seven to nine β-sheet structures. The diversity of superfamily members precludes generalization about

their role in tumor cell invasion and metastasis. However, the role of one family member seems straightforward. Vascular cell adhesion molecule-1 is an endothelial cell, cytokine-inducible, counter-receptor for VLA-4 (very late antigen-4) integrin, also known as $a_4b_1$ *integrin receptor.* The role of integrin receptors is discussed separately (see Role of Integrins in Tumor Invasion, later in this chapter). Normally, VLA-4 is expressed on leukocytes and functions in mediating leukocyte attachment to endothelial cells. VLA-4 is also found on tumor cells in malignant melanoma[44] and metastatic sarcoma but not in adenocarcinomas.[45] It is thought that expression of VLA-4 may facilitate interaction of circulating tumor cells with endothelium before tumor cell extravasation. This was demonstrated by intravenous injection of human melanoma cells into nude mice pretreated with VLA-4–inducing cytokines, which resulted in an enhanced number of lung metastases formed compared with no cytokine pretreatment of the mice.[46] Cell-cell adhesive interactions can either suppress or facilitate metastasis formation. Either role is dependent on the specific context and molecular mechanisms of cell-cell interaction.

## CELL–EXTRACELLULAR MATRIX INTERACTIONS IN TUMOR PROGRESSION

As already discussed, the interaction of the tumor cell with the ECM, in particular the basement membrane, defines the invasive phenotype, and tumor invasion is paramount to metastasis. It is now recognized that the ECM exerts a profound influence on cell behavior and that cells direct the assembly and disassembly of the matrix. This concept is known as *dynamic reciprocity* and also applies to the interaction of malignant tumor cells with the ECM. During the process of metastasis formation, malignant tumor cells must interact with a variety of different types of ECM. These types include the subepithelial basement membrane of the tissue of origin, stromal elements of the tissue of origin, subendothelial basement membranes during extravasation, and the stromal and basement membranes of the organ(s) at the site of metastasis growth. Attachment of nonneoplastic cells to the ECM is prerequisite for cell survival. A fundamental difference for neoplastic cells is the loss of anchorage requirement for cell survival and growth. The anchorage-independent growth of tumor cells may result from an uncoupling of cell survival signals transduced from the ECM by attachment, coupled with activation of cell-cycle progression that is associated with neoplastic transformation. Tumor cell interactions with the ECM have profound implications for both cell-cycle regulation and migration.

### ROLE OF CD44 IN TUMOR INVASION

CD44 is a transmembrane glycoprotein with a large ectodomain and single cytoplasmic domain. CD44 is involved in cell adhesion to hyaluronan (HA). The gene encoding CD44 is on the short arm of human chromosome 11[47] and contains both constant and variable exons.[48] As a result of this gene structure, a number of differentially spliced isoforms of CD44 can be generated. The isoform containing no variant exon sequences is referred to as *standard CD44.* A total of nine variant regions (v2 to v10) can encode protein sequences. Alternatively spliced messenger RNA variants of CD44 (CD44v) can contain one or more variant coding regions. More than 30 different splice variants have been detected by polymerase chain reaction analysis.[49] In addition to these variants, cell type–specific differences in glycosylation of the core protein also exist. The pattern of glycosylation and presence of variant exons influence the ability of CD44 to function in HA binding.[50]

Several lines of evidence suggest that CD44 expression plays a role in metastasis formation. Clinical correlation studies demonstrate that a variety of different types of cancer that express high cell surface levels of CD44 have a poorer clinical outcome when compared with tumors that have low CD44 surface expression.[51–53] Forced expression of CD44v4 to v7 confers metastatic ability to a nonmetastasizing rat pancreatic carcinoma cell line.[54] Metastasis formation could be blocked using antivariant antibodies.[55] However, the exact role of CD44v in metastasis formation remains elusive.

In some tumors, CD44-associated increases in tumor growth and metastatic potential correlate with CD44-mediated cell attachment to HA.[56–58] CD44 also functions in HA uptake and degradation that is also correlated with tumor cell behavior.[56,59,60] It also has been demonstrated that CD44 aggregation on the cell surface creates a binding site for the active MMP-9.[61] It is postulated that bound MMP-9 may liberate ECM-bound HA and facilitates tumor cell HA uptake and degradation. These findings link cell adhesion and ECM turnover. In addition, they suggest that CD44 may function at different stages of tumor cell invasion and metastasis and that the specific CD44 role may depend on the specific stage of metastasis formation that is examined.

### ROLE OF INTEGRINS IN TUMOR INVASION

Integrins are heterodimeric transmembrane proteins that are formed by the noncovalent association of α and β subunits.[61–64] Considerable redundancy within cell-ECM interaction mediated by integrins exist, because most integrins bind to several individual matrix proteins, and ECM components, such as laminin, fibronectin, vitronectin, and collagens, bind to several different integrin receptors. This fact suggests that integrins are capable of providing the cell with detailed information about the surrounding ECM environment, which is then integrated at the cellular level to generate a cellular response (Fig. 8-3). It is now well established that integrins can signal across the cell membrane in both directions.[65–67] Binding of ECM ligands to integrins is known to initiate signal transduction pathways that can result in cell proliferation, differentiation, migration, or cell death (apoptosis, anoikis). This is referred to as *outside-in signaling.* It is also known that intracellular events can modulate the binding activity of integrins for their ligands in the ECM, which is referred to as *inside-out signaling.* Both integrin clustering and ligand occupancy are crucial for the initiation of intracellular integrin-mediated signal pathways.[68]

Integrin-mediated signal transduction involves both direct activation of signaling pathways and collaborative signaling (also referred to as *cooperative signaling*), in which integrins modulate signaling events initiated through receptor tyrosine kinases.[67,69] The *cis* association of integrins with other receptors on the same cell surface results in formation of multireceptor complexes. There is little evidence that these multireceptor complexes signal through exclusive, integrin-specific pathways, but instead they cooperate with the other receptors to influence a variety of signaling pathways. Direct integrin signaling starts after engagement of the integrin with their ECM ligands, lateral clustering of the integrin receptors, and interaction of the integrin cytoplas-

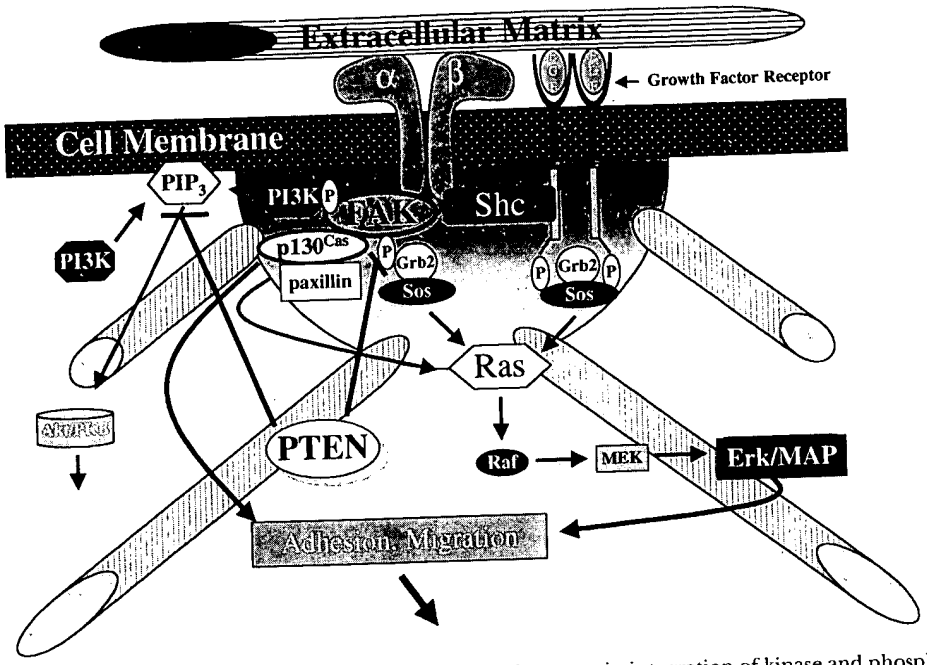


**FIGURE 8-3.** Role of integrins in tumor cell invasion and metastasis: integration of kinase and phosphatase activities. Binding of extracellular matrix components to integrin receptors initiates an intracellular signaling cascade that results in formation of a focal adhesion complex consisting of cytoskeletal and signal transduction molecules. Ligand binding to the integrin receptor results in integrin clustering and association of signal transduction molecules. Integrin receptor clustering induces autophosphorylation of focal adhesion kinase (FAK) on tyrosine 397. Subsequently, a Src homology 2 (SH2)-containing (Shc) adapter protein of the Src kinase family that binds to specific phosphotyrosine residues (Y397) on FAK is recruited to the integrin-FAK complex. Recruitment of additional proteins, such as α-actinin, talin, and paxillin, to this complex connects the focal adhesion complex to the filamentous actin cytoskeleton. Interaction of Shc with FAK results in additional sites of phosphorylation on the FAK molecule and subsequent recruitment of additional SH2 adapter proteins, such as Grb2 and the nucleotide exchange factor Sos. These interactions lead to activation of the mitogen activated protein (MAP) pathway, which stimulates tumor cell growth, adhesion, and migration. Similarly, receptors for growth factors can transiently associate with the focal adhesion complex to synergistically activate the MAP kinase pathway.

FAK activation also acts upstream of the Akt/protein kinase B (PKB) signaling pathway, which promotes cell survival. Association of the p85 subunit of phosphatidylinositol-3 kinase (PI3K) with tyrosine 397 in FAK mediates this effect. The rapid elevation of the phosphatidylinositol(3,4,5)triphosphate ($PIP_3$) lipid product of PI3K activity stimulates the Akt/PKB pathway, leading to enhanced cell survival.

Crk-associated substrate ($p130^{CAS}$) is another SH2- and SH3-conatining signal transduction that associates with FAK on integrin binding to the extracellular matrix. Interaction of $p130^{CAS}$ with FAK is mediated by a proline-rich region on FAK (residues 712–178) that interacts with the SH3 domain of $p130^{CAS}$. Activation of $p130^{CAS}$ promotes cell migration and invasion that is associated with enhanced metastatic behavior. The tumor suppressor gene PTEN inhibits cell adhesion, migration, and invasion. This inhibition is mediated by direct PTEN dephosphorylation of FAK and Shc, leading to negative regulation of the $p130^{CAS}$ pathway, which affects cell attachment, migration, and invasion. Down-regulation of the MAP kinase pathway by PTEN dephosphorylation of FAK and Shc negatively affects cell growth in addition to attachment and migration. PTEN is also known to directly dephosphorylate $PIP_3$ and negatively regulate the downstream Akt/PKB cell survival pathway. PTEN may also disrupt this pathway indirectly by dephosphorylation of FAK, which alters P13K activation. Thus, integrin-mediated regulation of cell growth, adhesion, migration, and invasion is a complex network of signal transduction cascades that have both positive (kinase) and negative (phosphatase) regulatory elements. EGF, epidermal growth factor; Erk, extracellular signal–regulated kinase; MEK, MAP kinase or ERK kinase; P, phosphorylated amino acid residual(s).

mic domain to form complexes with proteins that link to the cytoskeleton. This results in organization of cell structures known as *focal adhesions.*

Autophosphorylation of the focal adhesion kinase (FAK) was among the first integrin-mediated signaling events to be identified. This autophosphorylation event on tyrosine 397 results in recruitment of Src-family protein kinases that in turn results in phosphorylation of additional tyrosine residues on FAK. Phosphorylation of FAK can result in activation of the extracellular signal–regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathway. Activation of the MAP kinase pathway has been linked to induction of cell migration.[70] The activation of MAP kinase can be mediated by Grb-2 recruitment to FAK that then binds Sos, a guanine nucleotide exchange factor for Ras.[67,71] Alternatively, activation of the MAP kinase pathway can result from FAK phosphorylation of paxillin and the Crk-associated substrate ($p130^{Cas}$) that leads to binding of link proteins, such as Nck.[72] In addition, phosphorylation of FAK at tyrosine 397 creates a binding site for the regulatory subunit of phosphoinositol-3 kinase and triggers activation of this signal pathway.[73]

The knowledge that FAK is tyrosine phosphorylated on integrin activation suggests that focal adhesion–associated protein tyrosine phosphatases (PTP) could modulate Fak function. Several PTPs that interact with components of the focal adhesion

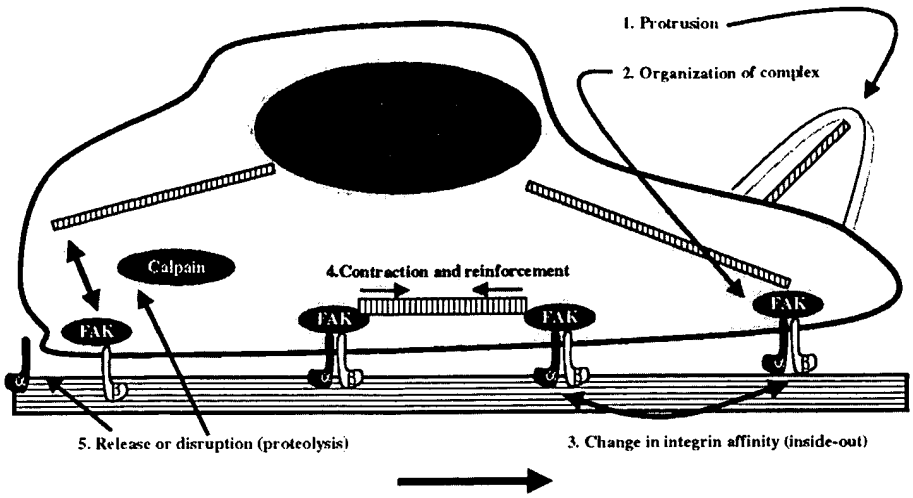


**FIGURE 8-4.** Cellular migration. Cell migration requires transmission of force from the extracellular matrix to the cytoskeleton. This is accomplished in distinct steps, several of which are illustrated. First, there is protrusion of a cellular projection (filopodia or lamellipodia), which is mediated by actin polymerization. The second step involves organization of the focal adhesion complex and connection with the actin cytoskeleton. This is accomplished via mechanisms described in Figure 8-3. Evidence suggests that if the integrin component of these complexes is not activated, these complexes are continually recycled across the cell surface without cell movement. Step 3 entails engagement of the integrin with the extracellular matrix, which is mediated by a change in integrin affinity. Traction force is generated and transmitted by the contraction and reinforcement of the actin cytoskeleton. The final step in cell locomotion is the release or disruption of the focal adhesion complex at the trailing edge of the migrating cell. Step 5 is accomplished by release of components of the integrin complex or via proteolytic disruption of focal adhesion kinase connections with the filamentous actin cytoskeleton.

complex have been identified. These are PTP-α and PTP-PEST [protein tyrosine phosphatase rich in proline (P), glutamic acid (E), serine (S), and threonine (T)], which negatively regulate Src and paxillin, respectively.[74,75] It has been shown that, in PTP-PEST–deficient cells, a defect in cell motility correlates with an increase in the size and number of focal adhesions.[76] This defect appears to be due in part to the constitutive increase in tyrosine phosphorylation of paxillin, as well as $p130^{CAS}$ and FAK.

A PTP that interacts directly with FAK is referred to as *PTEN*.[77] PTEN was identified as a tumor suppressor gene on human chromosome 10q23, which is frequently mutated or deleted in a wide variety of human cancers, including gliomas and prostate, breast, lung, bladder, endometrial, kidney, and oropharyngeal cancers.[71] The gene encodes both a phosphatase domain and also has extensive sequence homology to the cytoskeletal protein tensin. PTEN functions as a dual-specificity phosphatase in that not only is it a PTP but it also dephosphorylates the lipid phosphoinositol(3,4,5,)triphophaste ($PIP_3$). The current view is that both phosphatase activities function as tumor suppressor activities. The lipid phosphatase activity regulates levels of $PIP_3$, which in turn regulates activation of the protein kinase B pathway that is protective against programmed cell death (apoptosis, anoikis). As a PTP, PTEN regulates the phosphorylation status of FAK and Shc that in turn regulate cell adhesion, migration, cytoskeletal organization, and MAP kinase activation. To summarize, loss of PTEN function results in alterations in integrin-mediated signaling via FAK and $PIP_3$ pathways, which results in achievement of an invasive phenotype.[71] The protein kinase activity just described functions to promote cell invasion. This process is countered by the PTP activities, which act as tumor suppressors.

The roles of specific integrins in tumor progression and metastasis formation are dichotomous. The decreased expression of some integrins is associated with cellular transformation and tumorigenesis. These include $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins. HT29 colon carcinoma cells lacking $\alpha_5\beta_1$ expression are either significantly less tumorigenic or completely nontumorigenic when forced to express $\alpha_5$.[78] Loss of $\alpha_2\beta_1$ expression in breast epithelial cells correlates with the transformed phenotype, and reexpression of this integrin abrogates the malignant phenotype.[79] On the other hand, expression of some integrins directly correlates with tumorigenicity and tumor progression. For example, the $\alpha_v\beta_3$ integrin is expressed in metastatic melanoma but not in benign melanocytic lesions.[80,81] Antibodies against $\alpha_v$ integrins blocked growth of human melanoma xenografts in nude mice.[82] Integrin $\alpha_6$ expression is increased in oropharyngeal and bladder cancers and in lung tumors.[46,83–87] Tumor progression is associated with expression of $\alpha_3\beta_1$ in 82% of tumors. The molecular events associated with enhanced integrin expression and tumor progression are not well defined. Clearly, in nontransformed cells, integrins are implicated in growth regulation. But tumor cell growth is anchorage-independent. This observation suggests that the role of integrins in tumor invasion and metastasis may only be secondarily related to growth control. Integrins are also directly involved in cell migration.

## CELLULAR MIGRATION: NEW INSIGHTS

Cell migration requires transmission of propulsive force from the ECM to the cytoskeleton of the migrating tumor or endothelial cell (Fig. 8-4). Repetitive assembly of cytoskeletal elements to form membrane ruffles, lamellipodia, filopodia, and

pseudopodia accomplishes cell movement.[88,89] Lamellipodia are broad, flat, sheet-like structures in comparison to filopodia, which are thin, cylindrical projections. Cell movement begins with protrusion of a filopod or lamellipod. These are formed by polymerization of actin to form elongated central filaments in the filopod and a broader cross-weave mesh in the lamellipod.[90] At the leading edge of the protruding structures, integrins concentrate in specific regions, and after ligation with ECM, ligands form focal adhesions. These focal adhesions are anchored to the actin filaments.

Microinjection studies have shown that integrin association with the Rho family of guanosine triphosphate (GTP)ases is critical for organization and assembly of the actin cytoskeleton. It has been demonstrated that a hierarchical cascade exists among these GTPases that controls formation of specific cytoskeletal structures. Cdc42 and Rac control formation of filopodia and lamellipodia, respectively, whereas Rho controls stress fiber formation and focal adhesions.[91]

The integrin connection to the ECM provides adhesive traction, and contraction of the actin filaments results in forward propulsion of the cell body. As the cell moves, new projections occur at the leading edge and are anchored with new focal adhesions. As the cell moves forward, the focal adhesions appear to move in a retrograde fashion on the cell surface. This apparent movement of the focal adhesions has been observed using fluorescent-tagged beads coated with integrin substrates or antiintegrin antibodies.[92–94] These coated beads can also be used to actually measure the integrin-cytoskeletal traction forces generated, which has led to the demonstration that the strength of the integrin-cytoskeletal interaction can be modulated by the rigidity of the extracellular substrate. When cells are on a rigid substrate, more restraining force is exerted to prevent movement of the focal adhesion. Cells can detect this change in the substrate, and they respond by increasing the force generated by cytoskeletal linkage to the focal adhesion so that the cell pulls harder. This is referred to as *reinforcement of the integrin-cytoskeletal attachments.* Data suggest that Src kinases may be selectively involved in regulating this reinforcement.[94] Researchers have shown that beads binding to the fibronectin receptor in fibroblast containing either wild-type or Src-deficient cells show similar reinforcement of the fibronectin-receptor–actin cytoskeletal linkage. In contrast, when they use vitronectin, there is little reinforcement of the vitronectin receptor in wild-type Src-expressing cells, but a strong reinforcement in the Src-deficient fibroblasts. These authors also show selective association of kinase-defective Src–green fluorescent protein fusion with the $\alpha_v$ integrin subunit of the vitronectin receptor, but not with the fibronectin receptor. Possible interpretations of these results are that either Src is normally a selective inhibitor of reinforcement of force generation through the vitronectin receptor or that Src promotes the turnover of links between cytoskeletal and integrin components of the focal adhesion complex.

Investigators have used a $\beta_1$ integrin–green fluorescent protein chimera to follow focal adhesion cycling over the cell surface.[95] These experiments demonstrate that, in stationary cells, focal adhesions were highly motile and moved in a linear fashion to the cell center. In motile cells, the focal adhesions remained stationary and only moved at the trailing edge of the cell. These authors postulate a cellular "clutch-like mechanism" in which alteration in integrin affinity is seen in response to migratory stimulus. Regulation of integrin-ligand interactions by inside-out signaling helps determine the nature of cellular responses to the ECM, such as whether a cell becomes migratory or remains stationary.[96]

The last step in integrin-mediated cell migration is the release of the ECM integrin-cytoskeletal attachments at the trailing edge of the cell.[88] Two mechanisms have been identified in this detachment. The first involves the release of the integrins from the cell surface such that it is left on the substratum. Integrin release from the cell membrane has been observed in fibroblast migration[97] and by tumor cell lines *in vitro.*[98] A second mechanism that mediates release of the trailing edge of the cell is destabilization of cytoskeletal linkages intracellularly by either proteolytic activity or phosphatase activity. Calpain is a $Ca^{2+}$-dependent protease that localizes to focal adhesions and regulates retraction in Chinese hamster ovary cells migrating on fibronectin by destabilizing cytoskeletal linkages.[99]

Cell motility is a critical component of the invasive phenotype. Understanding the molecular mechanisms that confer tumor cell motility should allow identification of novel targets for disrupting this process and preventing tumor dissemination. Tumor cell motility can be correlated with metastatic behavior. When parameters such as pseudopod extension, membrane ruffling, or vectorial translation are measured, a quantitative increase in highly invasive and metastatic tumor cells is seen when compared with nonmetastatic counterparts. A variety of stimuli have been shown to stimulate tumor cell motility *in vitro,* including host-derived factors, growth factors, and tumor-secreted factors that function in an autocrine fashion to stimulate tumor cell motility. Autocrine motility factor is a 60-kD glycoprotein produced by human melanoma cells that stimulates tumor cell migration. Autocrine motility factor has been identified as neuroleukin/phosphohexose isomerase.[100,101] Autotaxin (ATX) is a 125-kD glycoprotein that elicits chemotactic and chemokinetic responses at picomolar to nanomolar concentrations in human melanoma cells. ATX contains a peptide sequence identified as the catalytic site in type I alkaline phosphodiesterases (PDEs), and it possesses 5-nucleotide PDE [EC 3.1.4.1 (Enzyme Commission designation for this enzymatic activity)] activity.[102–104] ATX binds adenosine triphosphate (ATP) and is phosphorylated only on threonine. Thr210 at the PDE active site of ATX is required for phosphorylation, 5-nucleotide PDE, and motility-stimulating activities. ATX also has adenosine-5-triphosphatase (ATPase) and ATP pyrophosphatase activities. ATX catalyzes the hydrolysis of GTP to guanosine diphosphate and guanosine monophosphate, of either adenosine monophosphate or inorganic pyrophosphate to inorganic phosphate, and the hydrolysis of nicotinamide adenine dinucleotide to adenosine monophosphate, and each of these substrates can serve as a phosphate donor in the phosphorylation of ATX. ATX possesses no detectable protein kinase activity toward histone, myelin basic protein, or casein. These results have led to the proposal that ATX is capable of at least two alternative reaction mechanisms—threonine (T-type) ATPase and 5-nucleotide PDE/ATP pyrophosphatase—with a common site (Thr210) for the formation of covalently bound reaction intermediates, threonine phosphate and threonine adenylate, respectively.[103]

## PROTEASES IN TUMOR CELL INVASION

As has been noted, matrix proteolysis has been recognized as a key part of the mechanism of tumor cell invasion. Tumor cells

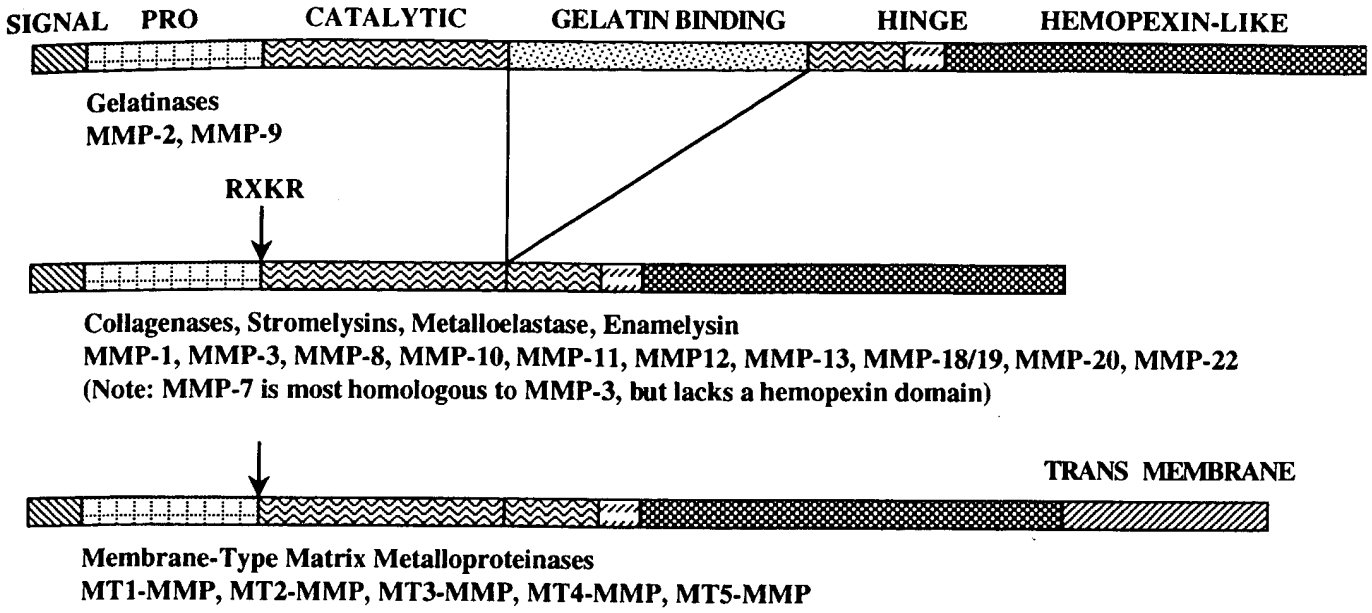


**FIGURE 8-5.** Simplified schematic domain structure of the matrixin family members. Most matrixins have the same basic domain structure, consisting of signal, pro, catalytic, hinge, and hemopexin-like domains. Matrix metalloproteinase 7 (MMP-7) lacks the hemopexin-like domain, and the gelatinases and membrane-type (MT) matrixins have additional domains. The arrow indicates the location of the furin RXKR cleavage site of MMP-11 and the MT matrixins. The length of each segment is roughly proportional to the number of amino acids that comprise the domain.

must be able to move through connective tissue barriers, such as the basement membrane, to spread from their site of origin. Although a variety of proteases have been implicated in this process, the family of proteases that has received the most attention has been the matrixins or MMPs. Matrixins and their specific inhibitors, the TIMPs, play important roles in normal physiology, because the ECM is a dynamic matrix of structural proteins, growth factors, and latent enzymes that is constantly being remodeled. Approximately 20 matrixins and four TIMPs have been characterized in humans and other animals.[105,106] The matrixins share a common domain structure (Fig. 8-5), although not all domains are represented in all family members. All of the enzymes have a signal peptide sequence; a propeptide domain (prodomain); a catalytic domain, which includes a highly conserved binding site for the catalytic zinc ion; and a hemopexin-like domain. Two family members, MMP-2 and MMP-9, have a gelatin binding domain containing three fibronectin type II repeats inserted into the catalytic domain just on the amino side of the active site sequence. Five family members have a carboxy-terminal transmembrane domain after the hemopexin domain. This subgroup is also known as the *membrane-type MMPs*. They reside on the cell surface, in contrast to the other family members, which are all secreted as proenzymes into the extracellular milieu. The TIMPs also share a high level of homology, including 12 absolutely conserved cysteine residues that are all involved in intramolecular disulfide bonds. TIMPs are divided into two domains by the disulfide-bonding pattern. An amino-terminal domain contains the inhibitory site, and a carboxy-terminal domain has other binding interactions. Because most of the MMP enzymes are secreted in their proenzyme forms, activation is a key regulatory step. Many of the matrixins are activated by an initial protease cleavage within the prodomain by another matrixin or by a serine protease, such as plasmin or urokinase-type plasminogen activator. This cleavage destabilizes the bond between a conserved prodomain cysteine sulfhydryl group and the catalytic zinc in the active site. The bond breaks and the prodomain is released, which frees the active site for catalysis. Unlike the other family members, which are typically activated outside the cell, MMP-13 and the membrane-type MMP subgroup are activated intracellularly by a furin-dependent cleavage of a conserved RXKR sequence that lies between the prodomain and the catalytic domain. The activation mechanisms have been the subject of a review by Murphy et al.[107]

An early indication of the importance of matrixins in tumor biology was the characterization in 1980 of a MMP secreted from a melanoma cell line that was able to degrade basement membrane collagen.[108,109] This finding was followed by numerous studies showing that secretion of matrixins enhanced tumor cell invasion in experimental model systems and that inhibition of protease activity by TIMPs or by synthetic metalloproteinase inhibitors impeded invasion. For example, the invasion of HT-1080 fibrosarcoma cells through Matrigel (a reconstituted basement membrane) is enhanced by addition of activated MMP-2 and inhibited by the addition of TIMP-2 or by zinc chelators.[110] *In vitro* evidence has been supported by the results of *in vivo* experiments using transfected cell lines. For example, when MYU3L bladder carcinoma cells are transfected with *mmp-2*, they have enhanced metastatic potential, whereas transfecting the highly metastatic LMC19 cell line with *timp-2* reduces its metastatic potential.[111,112] Mice genetically engineered to overexpress MMP-3 in breast epithelial cells develop

spontaneous breast carcinomas, possibly through the effects of MMP-3 on the E-cadherin/β-catenin system.[35]

Although these experiments and many others like them have demonstrated the key role of matrixin-initiated degradation in tumor invasion and metastasis, the role of these enzymes in this process is more complicated than an "degradation equals invasion" paradigm would suggest. Uninhibited matrix degradation would lead to complete dissolution of matrix proteins and would prevent tumor cells from being able to form attachments to each other or to matrix proteins, which is a necessary part of the tumor invasion mechanism. Thus, there is an implied balance between active proteases and inhibitors that results in an optimal invasive phenotype. As a demonstration of this principle, when A2058 melanoma cells are transfected with either sense or antisense *timp-2*, the invasive potential is decreased. Increasing TIMP-2 expression in this cell line enhances cell attachment and decreases motility, whereas decreasing expression decreases both cell attachment and motility. Thus, although protease action in tumor cell invasion is abnormal, it cannot be totally unregulated.

Matrixins have been shown to have effects other than removal of structural barriers to invasion. As noted above, some matrixins act on other proteins to reveal hidden activity. MMP-2 specifically degrades the γ2 chain of laminin-5, a structural protein in basement membrane, to reveal a site on the α3 chain that has chemotactic properties.[113] The physiologic role of this fragment may be to act as a wound-related chemoattractant; in tumors, however, this peptide may attract tumor cells to breaks in the basement membrane. In an analogous situation, MMPs have been shown to degrade plasminogen into angiostatin, the angiogenesis inhibitor.[114,115]

Research has focused on the interaction between cell surface adhesion molecules and the matrixin family. One obvious interaction is the simple degradation of cell surface adhesion molecules. For example, MMP-3 degrades E-cadherin on mammary epithelial cells, inducing an increased expression of vimentin and decreased expression of keratin.[34] The cell-matrix adhesion molecule CD44 is also cleaved by matrixins, permitting detachment from the matrix.[116] Decreased cell-cell or cell-matrix adhesion can be a proinvasive phenotype. However, beyond mere degradation of adhesion molecules, cells may control the scope of degradative activity by binding the soluble matrixins with cell surface adhesion molecules, thus limiting degradation to a zone in the immediate vicinity of the cell. On endothelial cells, the integrin $\alpha_v\beta_3$ binds MMP-2 through the matrixin's hemopexin domain in response to angiogenic stimuli.[117,118] Similarly, CD44 has been shown to bind MMP-9 to the surface of breast carcinoma and melanoma cells.[60] Interestingly, stimulation of melanoma cells by antibodies to CD44 increased expression of MMP-2,[119] suggesting that binding of matrixins to cell surface receptors might not only provide a mechanism for localizing protease activity but also may serve as an autocrine-stimulating loop mechanism for increased expression of matrixins.

## INTRAVASATION, EXTRAVASATION, AND ORTHOTOPIC EFFECT

The mechanisms of tumor cell intravasation have not been investigated as intensively as the other events in the metastatic cascade. This is due in part to the lack of suitable model systems. A clear role for protease activity in tumor cell intravasation has been demonstrated using the chick chorioallantoic membrane system.[120] In this model, human tumor cells are placed directly onto a chorioallantoic membrane in which the epithelium and basement membrane have been disrupted, allowing tumor cells direct access to the underlying connective tissue that is highly vascularized. Tumor cell intravasation is then quantitated by using polymerase chain reaction amplification of human-specific Alu genomic DNA sequences of tumor cells present in the chorioallantoic membrane on the other side of the chick embryo from the initial tumor cell inoculation. These experiments demonstrate that MMPs, as well as urokinase-type plasminogen activator and the urokinase-type plasminogen activator receptor, are involved in tumor cell intravasation.

Researchers[121,122] have used intravital videomicroscopy to study the events and mechanisms involved in tumor cell extravasation from the circulation. The results of these studies have profoundly changed our thinking about the metastatic process. It appears that a large number (80%) of circulating tumor cells remain viable in the circulation and extravasate up to 3 days after their introduction into the circulation. Surprisingly, both metastatic and nonmetastatic cells extravasate, and this process is not protease-dependent. However, only a small subset of cells (1 in 40) grow to form micrometastases, and even fewer (1 in 100) continue to grow, forming macroscopic tumors. Almost 40% of injected tumor cells remained as dormant solitary cancer cells. These findings suggest that the control of postextravasation growth of individual cancer cells is a dominant effect in metastatic inefficiency. More recent data suggest that the local environment of the target organ may profoundly influence the growth potential of extravasated tumor cells. The importance of tissue microenvironment (host) on growth of the primary tumor is well known and is referred to as *orthotopic effect.*[123]

Xenografts of human metastatic tumor cells in nude or SCID (severe combined immunodeficiency) mice infrequently recapitulate the behavior of the parent tumor to show a spontaneously metastatic phenotype. When injected intravenously, however, many of these same human tumor cells are capable of experimental metastasis formation in what are known as *lung colonization assays.* In pioneering experiments,[124–126] it has been demonstrated that this effect is due in part to important tumor-host interactions that are not usually provided by models using subcutaneous injection of tumor xenografts. Using orthotopic implantation of tumor xenografts, these investigators have demonstrated that the host microenvironment has a profound influence on a number of tumor cell parameters,[127] including tumor growth, invasive behavior,[126] response to chemotherapeutic agents,[128,129] growth factor and cytokine production,[130,131] and protease profiles for both urokinase and metalloproteinases.[132–134] An important host contribution to tumor progression is the frequent association of MMP production by stimulated stromal fibroblasts adjacent to invading tumor cells.[135]

During the 1990s, tremendous progress has been made in understanding the process of tumor cell invasion and metastasis formation, which is being revealed as a complex cascade of both promoting agents and suppressors. Loss of function (suppressor inactivation) may occur not only in the tumor cell but also in the host. Such tumor cell-host interactions are complex and difficult to examine in a controlled fashion. However, understanding these complexities is the future of research on cancer metastasis and tumor dissemination. The goal of this research will be the

development of new strategies for eradication of established metastases and prevention of new metastatic growth.

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